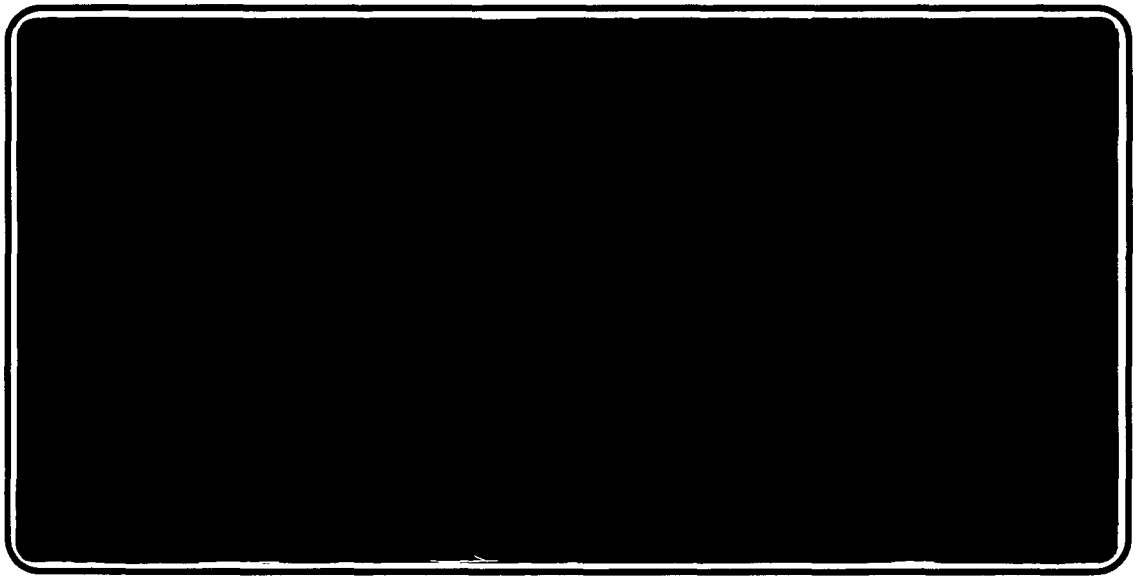
Institute of Paper Science and Technology
Atlanta, Georgia

IPST TECHNICAL PAPER SERIES



NUMBER 383

MATURATION OF DOUGLAS-FIR SOMATIC EMBRYOS IN SUSPENSION CULTURES

R. NAGMANI AND R.J. DINUS

JUNE, 1991

Maturation of Douglas-fir Somatic Embryos in Suspension Cultures

R. Nagmani and R.J. Dinus

Presented at
21st Southern Forest Tree Improvement Conference
Knoxville, Tennessee
June 17–20, 1991

MATURATION OF DOUGLAS-FIR SOMATIC EMBRYOS IN SUSPENSION CULTURES

R. Nagmani and R. J. Dinus[1/]

ABSTRACT

Utility of different CHO sources and levels for embryo maturation was evaluated with a Douglas-fir embryogenic culture. The original explant was an immature embryo from an open-pollinated seed orchard donor. Embryogenic tissues (250-300 mg (fw)) were moved from agar-based maintenance medium through a similar transition medium, containing 1% activated charcoal and lacking growth regulators, to 50 ml of liquid maturation media in 250 ml Erlenmeyer flasks. The maturation medium, a modified MS medium, contained half-strength micro- and macro-nutrients, included L-glutamine in lieu of ammonium nitrate, lacked growth regulators, and was supplemented with four sucrose, maltose, and glucose levels. One sucrose and one maltose level were tested with and without ABA. Cultures were incubated on a platform shaker (120 rpm); 16 hr photoperiod (soft-white fluorescent), 26 +/- 2°C. Cultures were transferred to fresh media, and embryo status was observed weekly.

Of the three CHO sources, only maltose advanced early-stage embryos present on maintenance and transition media to cotyledonary and/or mature stages. Glucose did not promote development to any significant extent. Sucrose had marginal effects with only one level, higher than routinely used in plant cell culture, moving small embryo numbers to advanced but still precotyledonary stages. All maltose concentrations moved numerous embryos to these stages within four weeks and, in turn, to maturity by the end of the six-week culture period. Embryo numbers, as well as morphologies, varied among maltose levels, with the lowest level (0.08M) maturing the largest number (82 per 50 ml of medium). Culture in liquid media also reduced the tendency of embryos to remain fused to one another as often occurs on agar-based media.

KEYWORDS

Pseudotsuga menziesii (Mirb.) Franco, embryogenesis, plant cell and tissue culture, suspension cultures, abscisic acid, carbohydrates, maltose.

ABBREVIATIONS

CHO = Carbohydrates, ABA = Abscisic Acid, MS = Murashige and Skoog, 2,4-D = 2,4-Dichlorophenoxyacetic acid, BA = 6-Benzylaminopurine.

[1/] Assistant Professor and Professor, respectively. Institute of Paper Science and Technology, 575 14th Street, NW, Atlanta, GA 30318.

INTRODUCTION

Somatic embryogenesis has been obtained in a variety of conifers since first reported for Norway spruce (Picea abies (L.) Karst) (Hakman et al. 1985) and European larch (Larix decidua Mill.) (Nagmani and Bonga 1985). Repeatable protocols, often with provisions for quantification, have been published for several species, e.g., Norway spruce (Becwar et al. 1987, 1988), white spruce (Picea glauca (Moench) Voss) (Hakman and Fowke 1987, Webb et al. 1989), white pine (Pinus strobus L.) (Finer et al. 1989), and coastal redwood (Sequoia sempervirens (D. Don) Endl.) (Bourgkard and Favre 1988).

Success with Douglas-fir and loblolly pine (Pinus taeda L.), however, has been limited, particularly with those steps involving embryo development and maturation. In earlier work with Douglas-fir, we obtained a few embryos, but many were fused together or otherwise abnormal (Nagmani et al. In press). A recent patent (Gupta and Pullman 1990) indicates that osmolarity of culture media plays a critical role in the maturation step. Addition of sugars, hexitols, or cyclitols capable of sufficiently raising osmotic potential greatly improved maturation of embryos of Norway spruce, Douglas-fir, and loblolly pine. In Douglas-fir, mature embryos and plantlets were obtained from a variety of genotypes. CHO source and ABA have also been tested in our laboratory. Substitution of maltose or glucose for sucrose in maturation media yielded fully developed loblolly pine embryos. Small but significant numbers were recovered, and all had normal phenotypes, morphologically and anatomically (Uddin et al. 1990).

These several findings prompted further investigation of CHO effects on somatic embryo maturation. This report describes results from preliminary experiments designed to extend our best loblolly treatments to Douglas-fir and to evaluate them in a suspension culture system.

MATERIALS AND METHODS

The embryogenic culture used in the present trial was initiated from an immature embryo in summer 1988 (Nagmani et al. In press). Developing open-pollinated cones from the seed orchard donor tree, WTC-570, were supplied by Weyerhaeuser Company. Initiation occurred on MSCG media (Table 1) supplemented with 5.0 mg/L 2,4-D and 2.5 mg/L BA.

Maintenance and proliferation were executed per Nagmani et al. (In press), with the following exceptions. Vigorous growth, especially after the first 1.5 years, required trial and error adjustment of the MSCG medium. Consistent performance was obtained by halving micro- and macro-nutrient concentrations and reducing the level of 2,4-D to 2 mg/L. The complete formulation (Table 1) is hereafter referred to as mMSCG.

Table 1. Basal media, supplements, and growth regulator combinations for initiation, maintenance, and maturation of Douglas-fir somatic embryos. Composition of MS media is shown for comparison.

Components, $mg \cdot L^{-1}$	MS	MSCG5/2.5	mMSCG2/0	mMSG[1/]
NH_4NO_3	1650	--	--	--
KNO_3	1900	100	950	950
$MgSO_4 \cdot 7H_2O$	370	370	185	185
KH_2PO_4	170	170	85	85
$CaCl_2 \cdot 2H_2O$	440	440	220	220
$Ca(NO_3)_2 \cdot 4H_2O$	--	--	--	--
KCl	--	745	--	--
KI	0.83	0.83	0.41	0.41
H_3BO_3	6.2	6.2	3.1	3.1
$MnSO_4 \cdot H_2O$	22.3	22.3	11.15	11.15
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	4.3	4.3
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.125	0.125
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.0125	0.0125
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.0125	0.0125
$NiCl_2 \cdot 6H_2O$	--	--	--	--
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8	27.8
Na_2EDTA	37.3	37.3	37.3	37.3
Inositol	100	100	100	100
Glycine	--	--	--	--
Nicotinic acid	0.5	0.5	0.5	0.5
Pyridoxine	0.1	0.1	0.1	0.1
Thiamine HCl	0.1	0.1	0.1	0.1
Sucrose	30,000	30,000	30,000	--
Glutamine (G)	--	500	500	500
Casein Hydrolysate (C)	--	1000	1000	--
Agar	0.8%	0.8%	0.8%	--
Growth Regulators				
2,4-D	--	5	2	--
BA	--	2.5	--	--
Kinetin	--	--	--	--
ABA	--	--	--	2.6

[1/]See text for CHO sources and concentrations used in mMSG maturation medium.

Maturation was accomplished in two steps: a transition step to remove growth regulators used for maintenance and proliferation, and the actual maturation step in which various CHO and ABA treatments were tested. The first, or transition, step involved incubation in the dark for seven days on mMSCG supplemented with 1 percent activated charcoal and gelled with 0.8

percent agar. All cultures were treated the same during the maintenance and transition steps to ensure consistent composition before transfer to experimental maturation media.

The second, or maturation, step involved incubation on modified mMSCG medium. Modifications consisted of not adding casein hydrolysate and using a liquid, rather than gelled, form. The formulation (Table 1) is hereafter referred to as mMSG. To evaluate CHO effects, mMSG was supplemented with four different levels each of sucrose (control) and maltose (0.08, 0.17, 0.31, and 0.49 M) and glucose (0.16, 0.33, 0.49, and 0.66 M).

To glimpse ABA effects, 0.08 M sucrose and maltose treatments were tested with and without ABA (10 µM). This level of sucrose (3 percent w/v) is used widely in plant tissue culture systems, can be regarded as a control or standard, and was therefore considered a logical starting point for testing growth regulator supplements.

Weighed masses of embryogenic tissues (250 to 300 mg (fw)) were transferred from maintenance medium first to transition medium, and then dispersed in 50 ml of mMSG maturation medium in 250 ml Erlenmeyer flasks. Cultures were incubated on a platform shaker (120 rpm) in a lighted culture room; 16 hr photoperiod (soft-white fluorescent) and 26 +/- 2 °C. Sub-culturing was done every seven days.

Individual treatments were represented by four flasks (replications). One flask from each treatment, chosen at random, was used to monitor development. A 3 ml aliquot was withdrawn from each such flask every seven days for microscopic observation. Embryo stages and numbers per stage were recorded. Stage identification followed the convention of Buchholz and Stiemert (1945). Observations from all flasks were taken at the end of the overall experiment, except for the few lost to contamination. The experiment was terminated at six weeks as treatments yielding responses had produced mature somatic embryos by that time or earlier.

RESULTS AND DISCUSSION

Embryos present in tissues on maintenance medium were at Stage 1, the very earliest stage of development (Figure 1A). Incubation on maintenance and transition media solidified with agar generally resulted in fusion of two or more embryos (Figure 1B). In earlier work (Nagmani et al. In press), this same phenomenon was observed whenever solid media were used. Even after transfer to liquid maturation medium (mMSG) in the present research, embryogenic tissues were slow to disassociate. By the first formal observation date, seven days after transfer to this medium, however, microscopic examination showed numerous embryos clearly free of one another (Figure 1D and E).

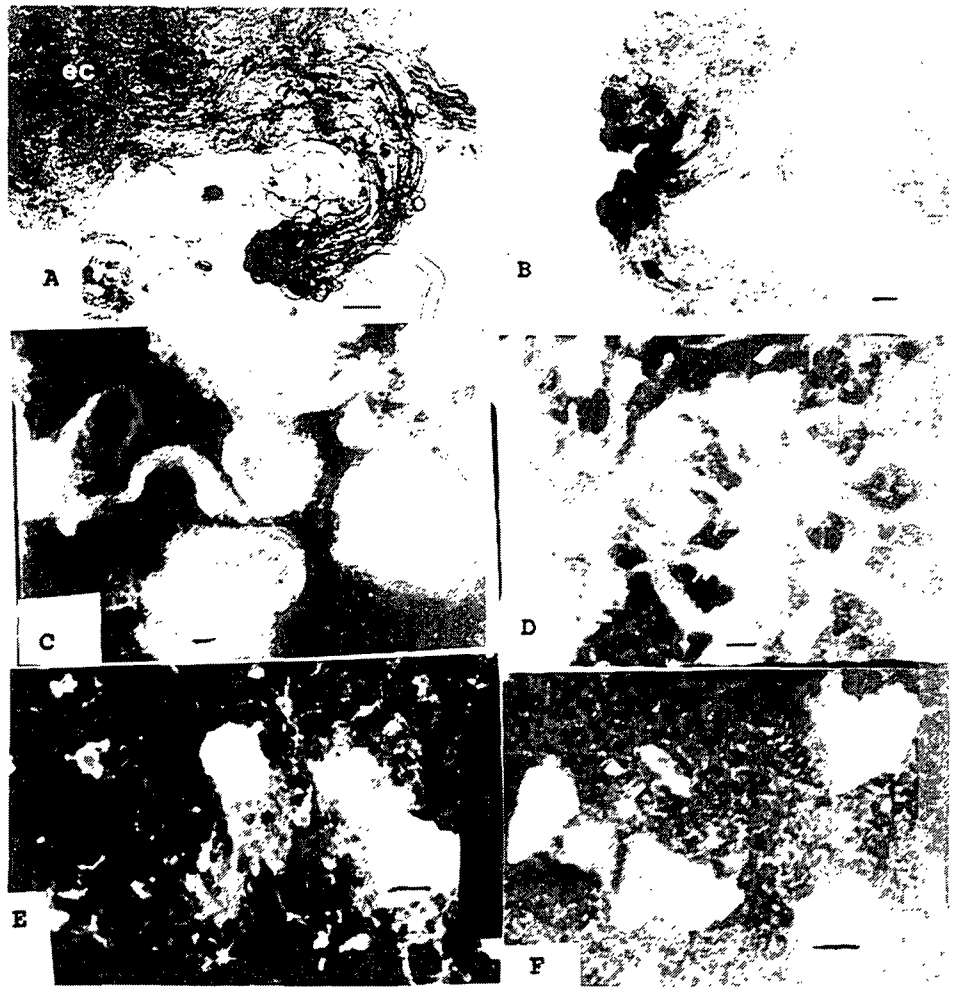


Figure 1. Maturation of Douglas-fir somatic embryos.

A. Early stage embryo (Stage 1) on agar-based maintenance medium. Scale bar = 100 µm.

B. Small group of embryos adhering together on agar-based transition medium. Scale bar = 50 µm.

C. Stage 2 and 3 embryos after 4 weeks in liquid maturation medium containing 0.31 M sucrose. Scale bar = 1 mm.

D.,E. Stage 2 and 3 embryos after 4 weeks in liquid maturation medium containing 0.08 M maltose and 10 µM ABA.

F. Mature cotyledonary embryos (Stages 5-7) after 6 weeks in maturation medium supplemented with maltose but without ABA. Scale bar = 1 mm.

Of the three CHO sources, only maltose advanced embryos to the cotyledonary and mature stages (Table 2). Glucose did not promote development to any significant extent. As in past trials (Nagmani et al. In press), sucrose effects were marginal. Incubation with 0.31 M sucrose moved a small number of embryos to, but not beyond, Stage 3, a still precotyledonary stage (Figure 1C). These may have developed further, but this and several related treatments became contaminated and were terminated at the end of the fourth week. In view of these and our earlier findings with sucrose, higher than standard concentrations of sucrose and/or glucose warrant reexamination.

Table 2. Maturation of Douglas-fir somatic embryos after 4 and 6 weeks in suspension cultures supplemented with various CHO concentrations with and without ABA.[1/]

Treatment Combination		Weeks in Culture:	4	6
Maltose	+/- ABA	Embryo Stage:	2-3	5-7
(M)	(10 µM)		--- Mean No. per 50 ml ---	
0.08	+		151	2/
0.08	-		190	82
0.17	-		145	55
0.31	-		155	60
0.49	-		185	49

1/ Sucrose and glucose supplements moved only a very few embryos to stages beyond those observed on maintenance medium.

2/ Treatment terminated at four weeks as a result of contamination.

In contrast to sucrose, and regardless of concentration, maltose moved numerous embryos to Stages 2 and 3 by end of the fourth week (Table 2). Many of these, in turn, were advanced to Stage 5 and/or beyond by the sixth week. Relative numbers and morphology varied among maltose concentrations. As an example, the lowest concentration (0.08 M) produced the largest numbers, but many were green and had elongating radicles (Figure 1F) - a potential symptom of precocious germination.

Addition of 10 µM ABA along with 0.08 M maltose produced similar effects by the end of the fourth week. Fate of embryos exposed to ABA, regrettably, could not be observed beyond that point, as contamination forced early termination of the treatment. We speculate that they would have developed further and that precocious germination would not have occurred.

Findings from these preliminary experiments with Douglas-fir suggest, at least for this one genotype, that maltose fosters somatic embryo maturation, that ABA may also play an important role, and that suspension cultures offer significant advantages over solid media. Results concerning maltose and ABA agree with our earlier work on loblolly pine (Uddin et al. 1990), where maltose (or glucose) in combination with ABA was superior to sucrose. Whether

these alternative sugars are better energy sources than sucrose, regulate media osmolarity, or act by some other mechanism remains uncertain. According to Gupta and Pullman (1990), timed addition of selected CHO additives adjusts osmotic potential of culture media, thereby governing course and pace of development. Future research, involving an array of genotypes, should seek to confirm the best CHO additives, better define needed concentrations, clarify advantages of liquid over solid media, and determine mechanisms underlying such modifications.

ACKNOWLEDGMENT

The authors wish to acknowledge the financial support and technical advice provided by member companies of the Institute of Paper Science and Technology.

LITERATURE CITED

Becwar,M.R., T.L.Noland, and S.R.Wann. 1987. A method for quantification of the level of somatic embryogenesis among Norway spruce callus lines. Plant Cell Reports. 6:35-38.

Becwar,M.R., S.R.Wann, M.A.Johnson, S.A.Verhagen, R.P.Feirer, and R.Nagmani. 1988. Development and characterization of *in vitro* embryogenic systems in conifers. P. 1-18 *in* Somatic cell genetics of woody plants, M.R.Ahuja (ed.). Kluwer Academic Publishers, Dordrecht, The Netherlands.

Bourgkard,F. and J.M.Favre. 1988. Somatic embryos from callus of *Sequoia sempervirens*. Plant Cell Reports. 7:445-448.

Buchholz,J.T. and M.L.Stiemert. 1945. Development of seeds and embryos in *Pinus ponderosa* with special reference to seed size. Illinois Academy of Science Transactions. 38:27-50.

Finer,J. J., H.B.Kriebel, and M.R.Becwar. 1989. Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.). Plant Cell Reports. 8:203-206.

Gupta,P.K. and G.S.Pullman. 1990. Method for reproducing coniferous plants by somatic embryogenesis. U.S. Patent No. 4,957,866. 14 p.

Hakman,I., L.C.Fowke, S.von Arnold, and T.Eriksson. 1985. The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). Plant Science. 38:53-59.

Hakman,I. and L.C.Fowke. 1987. Somatic embryogenesis in *Picea glauca* (white spruce) and *P. mariana* (black spruce). Can. J. Bot. 65:656-659.

Nagmani,R. and J.M.Bonga. 1985. Embryogenesis in subcultured callus of *Larix decidua* L. Can. J. For. Res. 15:1088-1091.

Nagmani,R., M.A.Johnson, and R.J.Dinus. In press. Effect of explant and media on initiation, maintenance, and maturation of somatic embryos in Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir). In Woody plant biotechnology, M.R.Ahuja (ed.) (Proc. IUFRO Somatic Cell Genetics Working Party and NATO Advanced Research Workshop on Woody Plant Biotechnology, Placerville, CA, Oct. 15-19, 1989.). Plenum Publishing Corp., New York.

Uddin,M.R., R.J.Dinus, and D.T.Webb. 1990. Loblolly pine somatic embryo development and maturation. Abstract in IUFRO World Congress Proc., Montreal, Canada.

Webb,D.T., F.Webster, B.S.Flinn, D.R.Roberts, and D.E.Ellis. 1989. Factors influencing the induction of embryogenic and caulogenic callus from embryos of Picea glauca and P. engelmanii. Can. J. For. Res. 19:1303-1308.